FILE

212409

STIC-ILL

From: Schmuck, Jill
Sent: Monday, April 27, 1998 4:37 PM
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Thanks!!

L1 ANSWER 5 OF 5 MEDLINE
AN 87098839 MEDLINE
DN 87098839
TI Primary cultures of various differentiated human cells and their transfer.
AU Yamane I
SO GAN TO KAGAKU RYOHO [JAPANESE JOURNAL OF CANCER AND CHEMOTHERAPY], (1987 Jan) 14 (1) 211-9.
Journal code: 6T8. ISSN: 0385-0684.
CY Japan
DT Journal; Article; (JOURNAL ARTICLE)
LA Japanese
FS Priority Journals; Cancer Journals
EM 198704

AU1H 4/29 SY MS

=> d l1 5 ab

L1 ANSWER 5 OF 5 MEDLINE
AB From our long experience with primary cultures of ***human*** ***differentiated*** ***cells*** , we have been able to come to the following conclusions. The culture media routinely employed have been developed using established cell lines but not primary cells as the growth marker. Therefore the culture media for primary cultures should be modified from those routinely employed. In addition the concentrated supplementation of serum to basal media dose not contribute to the growth of primary differentiated cells, and in fact on the contrary, is advantageous for the growth of non-target fibroblasts which would hamper the growth of the target cells. A hypoxic culture environment is more favorable for primary cultures especially when smaller cell numbers are used as the inoculum. The primary culture cells are more fragile in comparison with established cell lines. Therefore, the low-temperature cell diopersion procedure is recommended with the use of diluted crystalline trypsin saline. On the basis of the above findings, we have successfully carried out primary and transfer cultures of human esophageal and gall bladder epithelial cells, skin keratinocytes and endothelial cells. Their epithelial mature was proved by the presence of keratin in their cytoplasm, and the phenotype of endothelial cells was evident from the presence of Factor VIII-related antigen in their cytoplasm. Culture of endothelial cells requires the supplementation of a specific growth factor which we have utilized and isolated from conditioned medium of human

CAL 4/30 M

(連載講座)

ヒトの分化細胞の初代培養と継代

——血清添加よりの逃走——

山根 績*

要旨 著者らの長年の経験に基づいて，ヒトの上皮細胞等の分化機能をもつ細胞の初代培養につき述べた。まず培地に関して，現在常用の培地はほとんど樹立株細胞の増殖を指標として開発されたもので，初代培養用のものでないこと，このような培地に血清を高濃度に添加したものを使用しても決してヒトの上皮細胞等を効率的に初代培養できないのみならず，分化細胞の増殖の障害となる線維芽細胞の優勢増殖をもたらすことが多いこと。培養ガス環境として，大気中の酸素分圧よりも低く，生体内のそれに近い低酸素分圧の方が，とりわけ接種細胞数が少ない場合に，大気の酸素分圧よりも細胞増殖が旺盛であることを強調した。また生体内の細胞は株細胞と異なり，単個細胞の分散に用いられるトリプシン等のタンパク分解酵素の作用に弱いので，低温で薄い結晶トリプシン液を用いることが望ましいことを述べた。

このような知見に基づき，ヒトの，食道上皮，皮膚表皮，胆嚢上皮および（上皮細胞でないが）臍帯静脈血管内皮の各細胞を，無血清培地を用いた初代培養とその継代培養に成功した。各上皮細胞は細胞質内のケラチンの存在等でその上皮性が証明され，血管内皮細胞の培養では細胞質内に血液凝固第八因子抗原の存在で内皮細胞性が証明された。ヒトの血管内皮細胞の培養には特殊の成長因子を要するが，著者らはこれをヒトの二倍体線維芽細胞の培養ろ液より得て培養に供するとともに，精製単離した。これを数ナンノグラムを家兎眼球角膜内にポリアクリルアミドとともに接種すると，約2週間で大小多数の血管を角膜内に新生し成長した。この血管は直径数ミリにまで達したが，組織切片染色標本から内皮細胞のみよりなる静脈洞的性格のものであることが判明した。

〔癌と化学療法 14(1)：210-219，1987.〕

はじめに

動物細胞の培養の歴史をたどってみると，もともと細胞培養の研究はすべて動物の体内で生育している細胞の培養，すなわち初代培養を用いた研究であった。ところが1942年 Sanford らによって C3H 系マウスの皮下線維芽細胞由来の L929 細胞，および1950年代初当 Gey らによってヒトの子宮頸部がん由来の HeLa 細胞等の株細胞が樹立されて以来，これ等の株細胞の培養を利用した細胞の増殖，遺伝，分化に関する研究が数多く現われるようになった。

しかしいわゆる株細胞は元来生体内に存在したごく一部の特定の細胞が長期間淘汰されながら残存生育を続け，また長年の長期継代培養の間に多くの変異を受けながら維持されてきたもので，もともと生体内に存在した普通の細胞とは形質的にかなり異なった特殊な細胞であるといえるのではあるまいか。多くの株細胞と生体内で生育している細胞と異なると思われる性質をあげてみると，単層増殖性の細胞では細胞分散に用いられるトリプシン等のタンパク分解酵素に対して耐性というか寛容性が高いことであろう。樹立株細胞の培養を分散して継代培養する場合でも，むやみに長期間細胞分散に用いるトリプシン等の酵素液に培養を接触させると細胞が障害され，継代培養がうまくいかないことは私どものたまたま経験するところであるが，生体内細胞の初代培養では特にこの

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点に留意しなければならないことも私どもの経験的に熟知していることである。これは株細胞が長年の長期継代培養において各継代ごとに培養に用いられるトリプシンに耐性の細胞を淘汰選択して継代し続けた結果，特殊なトリプシン耐性株になったのにほかならないのであろう。

つぎに生体内で生育している細胞のガス環境が株細胞の試験管内で培養されている環境と著しく異なることをあげたい。大気中の酸素分圧は約19%であるが，生体組織中の酸素分圧は組織によっても異なるが大気中のそれの1/3ないし1/5である[1]。樹立株細胞が大気中の酸素分圧下でも旺盛に増殖するのは，生体中よりもより高い酸素分圧の大気中に長年馴養される間に適応ないし変異したものであろう。現在多くの細胞の培養に（多く）用いられている培地は株細胞の増殖を指標として確立されたものである。しかし前述のように株細胞は生体中の多くの細胞が淘汰され，一部の特定細胞が生残し，さらに試験管内で変異を重ねて樹立されたもので，生体内で多数を占める細胞とは栄養要求上でもかなり異なっていると考えられる。

以上体内で生育している細胞の培養すなわち初代培養の，株細胞培養と異なる条件を羅列してきた。私どもはヒトの上皮細胞や血管内皮細胞等分化機能をもった細胞の初代培養とその継代の研究を約十年間行ってきたが，この研究から得られた共通の結果について，株細胞の培養と対比できる問題点について本稿で述べてみたい。

I. 培地の検討

上述のように現在広く用いられている培地の多くは株細胞の増殖を指標として，しかも（透析）血清を培地添加した条件で検討されて来たものである。しかし私どもの長年にわたる経験では上皮性細胞の初代培養の場合2%以上血清を基礎培地に添加しても（後述の）一般的成長因子を添加している限り，（2%以上の血清添加は）上皮性細胞の増殖には全然寄与しないことが明らかになった。このことは私どものみならず Ham ら[2]や Lechner[3] もすでに指摘しているところである。したがって血清添加を前提として開発された既成の培地は再検討が必要であることも当然であろう。

私どもはヒトの二倍体線維芽細胞[4]，吉田肉腫[5]，ヒトの血管内皮細胞[6]，ヒトの食道上皮細胞[7]等の初代培養の成績を検討した。その際出発の基礎培地としては MEM を用いた。その理由はこの培地の確立に至るまでアミノ酸，ビタミン等に対する栄養要求が定性的，定量的に仔細に検討された成績が Eagle 一門により報告されている唯一の培地であるからである。勿論彼らは5～10%に透析血清を添加した培地を用いたことはいうまでもない。私どもは上記細胞の初代培養の成績から，MEM の培地成分の他にさらに下記の低分子物質が必要であることを見いだした。

1. アミノ酸

MEM に含まれているアミノ酸の他にセリン（小数細胞培養にこれが必要なことを Eagle 一門も夙に報告），グリシン，プロリンおよび大量のグルタミン酸（無血清培養の場合グルタミンの増量を必要とした報告が見られるが，下記のようにプリン，ピリミジン類を添加している限り，規定量のグルタミン酸の添加で十分である。）

2. ビタミン

イノシトールおよびコリンの量を MEM 記載の5～10倍量添加する。極微量のビオチンおよびビタミン B_{12}，細胞によってエタノールアミン。コリン，イノシトール，エタノールアミンはセリンとともにビタミンとはいっても脂質の構成成分として必須なものである。

3. プリン，ピリミジン類

これらの物質は MEM 成分には含まれていないが完全無血清培養の場合には必須で，一般には少量のプリン塩基（例ヒポキサンチン）と微量のピリミジヌクレオシド（例チミジン）の組み合わせが必要である。

4. 無機塩

MEM には1mM のカルシウムが加えられているが，表皮細胞や食道上皮細胞のような重層上皮細胞および膀胱上皮，前立腺，気管支上皮などの細胞ではカルシウムの培地添加量を MEM のそれの1/10以下に減量することが必要である。その他鉄，微量の亜鉛および超微量のセレン等が必須である。鉄については特に重要であるが，成長

因子の項でふれることにする。

5. 脂肪酸

オレイン酸，リノール酸，アラキドン酸等の高級不飽和脂肪酸が必要であるが，これらの脂肪酸は水に不溶であるため，何らかの水和性担体に結合させて培地添加することが必要である。もっともこれらの脂肪酸をそのリポゾームの形で培地添加している報告もあるが，リポゾームは不安定で容易に大きなミセルを形成するので不適当である[8]。

6. 成長因子

G. Satoら[9]はすべての細胞の増殖には共通にインシュリン，トランスフェリンおよびセレンという因子が必要であると提案した[9]。私もこの提案に原則的には賛成であるが，このうちセレンは無機物質であって成長因子ではない。またインシュリンやトランスフェリンはきわめて高価で経済的に使用困難であるという人もいるが，インシュリンは国産の結晶ウシインシュリン（清水製薬製）で1g5万円程度で，必要量は培地1l当り1mgなので，コストは50円/lとなり，遺伝子組換えのヒトインシュリンが普及すれば，少なくとも現在のコストの半分位下になると考えられるので，培地1lのコストはグルタミン等のアミノ酸とほとんど変わらないことになる。トランスフェリンに関して私どもはある種の安価な鉄キレート剤と大量の塩化第二鉄とをキレートさせて細胞にあたえると，トランスフェリンの代替となることを見出した[10]。この方法を用いるとトランスフェリンの価格は問題にならなくなると思う。

つぎに各細胞に特異的に必須な成長因子をみいだすことは実際には相当面倒なことである。私どもはウシの脳の抽出液をヒトの表皮，食道肝実質細胞，胆嚢等の上皮細胞の無血清初代培養に用いてその有効性を確かめている。勿論抽出液は化学的にはまったく粗成であり，またその有効因子も決して単一なものではなく，種々の有効因子の混合物であると考えられるが，胎児牛血清を10%も培地添加する場合に比べて，上記の上皮細胞の分離培養の邪魔になる出発資料混在の線維芽細胞の優勢増殖をもたらし難い点で明らかに有用である。

ここで私どもの用いているその抽出法の概要について述べる。この抽出法は原則的には T. Maciag らがウシの脳視床下部から血管内皮細胞成長因子（ECGF）の抽出で用いた方法を略踏襲したものであるが[11,12]私どもの経験では脳視床下部だけでなく，脳全体から抽出しても有効因子の得られることが認められている。まずウシの脳を取り出し，これと同量の（−）PBS を加えて，4°C でホモジナイズして遠心する。遠心上清に0.5%の割合にストレプトマイシンを加えて攪拌し，上清に混在している脂質（および DNA，RNA）を複合体として沈澱させ，その遠心後の上清を（ストレプトマイシンと無機塩を除くため）低温下で透析する。初代培養出発材料に線維芽細胞の混在が多い場合には上記抽出液を Sephadex G 100のカラムを通過させ，その比較的低分子分画を用いることにしている。この脳抽出液は1～2%の割合に無血清培地に添加して用いる。

つぎに私どもがヒトの血管内皮細胞の初代培養の培地に添加するヒト二倍体線維芽細胞の conditioned medium について述べてみたい[13,14]。もともとある細胞を feeder layer として他の培養困難な細胞を培養しようとする試みはしばしば用られているが，さらに前者の細胞の conditioned medium を加えた培地を用いて後者の難しい細胞を培養しようとする試みもよく知られている。私どもはヒトの二倍体線維芽細胞中にヒトの二倍体血管内皮細胞の増殖を著しく促進——血清(10%)培地に一般の成長因子を加えても，この因子がなければ増殖しないほど必須的に促進——することを見出して，血管内皮細胞の初代培養のみならずその長期継代に用いている。その使用量は培地と等量または半量である。なおこの有効因子の精製とその生物活性については後でふれる。

II. 培養ガス環境

大気中には20%弱の酸素が含まれているが，動物臓器の組織にはその1/3ないし1/5量の酸素しか含まれていない（図1）[1]。つまり生体中に生育している細胞は大気中よりも著しい低酸素分圧中で生育しているわけである。それゆえ生体中から分離された細胞は樹立株細胞と異なり低酸素分圧下で培養した方が，むしろより旺盛に増殖したり，

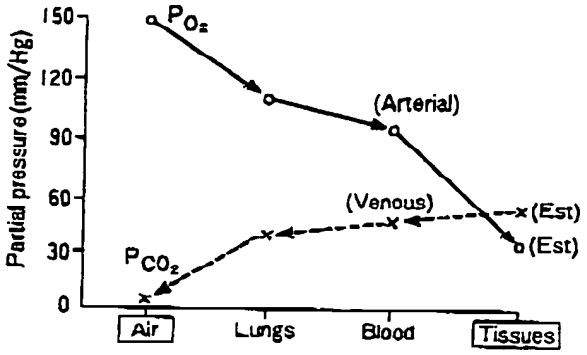

図 1 生体内における酸素（および二酸化炭素）の分圧（Rev. of Med. Physiol. より引用）

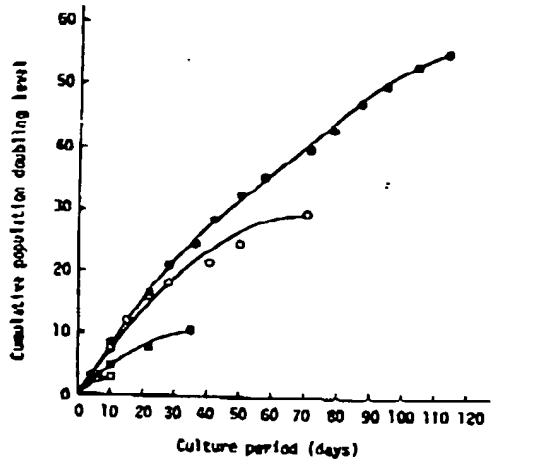

図 2 ヒト血管内皮細胞の培養ガス環境による長期継代の格差

より長期間体外で保持される可能性があるはずである。事実私どもはヒトの臍帯静脈由来の血管内皮細胞を長期継代培養する場合にも，大気ガス環境（勿論5% CO_2 を含む）で継代培養した場合と，7%の割合に酸素を含む低酸素ガス環境で継代した場合とを比較してみると，低酸素環境で継代した方が明らかにより長期に継代できることが判明した（図2参照）。このことは大気の酸素分圧下ではより多く細胞の発生する super-oxide や per-oxide によるものと考え，培地中に DTT 等の酸化還元電位低下の還元剤やカタラーゼ，super oxide dismutase(SOD)，ヘモグロビン，セルロプラスミン，セルロプラスミン，ハプトグロビン等を加え，大気酸素分圧下でヒトの二倍体線維芽細胞を培養すると，低酸素分圧下と同様に無血清条件で増殖する。しかしこれを長期継代培養すると上記の因子を加えただけでは（無血清条件では）大気中では長期培養はできなかった。

私どもはヒトの血管内皮細胞のみならず，ヒトの皮膚表皮[15]，食道[16]，胆嚢[17]等の上皮細胞を無血清条件で長期培養を行う場合低酸素ガス環境でこれが可能なことを明らかにした。勿論血清添加培地を用いる場合にはさほど顕著な差異はみられなかったが，それでも二倍体細胞（がん細胞でなくて）の長期培養の場合には低酸素ガス環境下で培養した方が明らかに安定した培養成績が得られたことはいうまでもない。

III. 低温細胞分散

生体内で生育している細胞は上述の理由で樹立株細胞と異なり，培養細胞の分散に用いられているトリプシン等のタンパク分解酵素にその細胞表面が弱いと考えられる。ところが（結晶）トリプシンは他の酵素と異なり，低温でもある程度有効であるとともに，低温でやや長い間作用させてもミトコンドリア等の細胞の内部に到達せず，細胞表面に留まっていることが電子顕微鏡的に明らかにされている。そこで Ham ら[19]は低濃度の結晶トリプシンを低温で作用させ，多く細胞の無血清培養に成功している。私どもは彼らの方法を略踏襲してヒトの分化細胞の初代培養とその継代に成功している。私どもの行っている手技の大略を述べると，最初（−）PBS で単層培養を洗ったのちトリプシン液（300μ/ml）を添加後，数十秒処理，培養の上にたまったトリプシン液をピペットで除去して数分間（これは細胞によって多少異なる）放置，つぎにピペットフラッシュしつつ細胞を剥離した後，これを数回洗滌して培養に供する。この際トリプシン処理後，必要があれば大豆トリプシン阻止因子（STI）液で一旦細胞を処理して後，培養容器に接種する。

なお一般にトリプシンは上皮細胞に対してよりも，線維芽細胞の培養に対してより有効に容器基質から細胞を剥離する性質があるので，短期間トリプシン液を初代培養にかけてこれに混在する線

維芽細胞（のみ）を半ば選択的に剝離して，上皮細胞だけを基質上に残せる可能性がある。もっともこれは程度問題であり，混在する線維芽細胞を完全に除こうとする，目的とする上皮細胞も剝離するので，1回だけの操作では不十分な選択的剝離に終わることを承知の上で，継代ごとに数回くり返す方がよい。また混在する線維芽細胞の除去には顕微鏡下に機械的にこれを剝離することが時として有効なこともある。

IV. ヒト食道上皮の初代培養と継代[7]

培養材料として40歳から77歳までの食道ガン患者の手術摘出材料から正常食道部を分離して用いた。この際にドゴール液で染色して正常食道部と腫瘍部位とは区別して，腫瘍部より十分離れた正常部位のみを材料として用いた。その上皮部を皮下部からメスで剝離して上皮部を用い供した。この上皮部はメスで細切され，EDTA を含むトリプシン溶液で60～90分消化され，得られた遊離細胞を培養に用いた。

この培養に用いた培地は私どもの開発した無血清培地 RITC 80-7 の基礎培地の組織中の塩化カルシウムを除くか，基本量の1/50量に減量したものに牛アルブミンVを5 g/l と上述のウシの全脳抽出物（凍結乾燥したもの）を100mg/l の割合に添加して用いた。まずⅠ型コラーゲン（市販）でプラスチックシャーレをコートしたものに細胞を接種した。その増殖カーブを図3に示す（図3参照）。この方法でヒトの正常食道上皮細胞を初代培養から継代培養すると，17～18 PDL まで4回位移植継代培養が可能である。勿論培養された細胞はサイトケラチンに対する螢光抗体で染色すると細胞質内に枝状ないし葉状のケラチンが多く確認され，また電子顕微鏡像で細胞間のデスモゾームの存在が認められた上皮性の細胞であることが証明されている（図4参照）。

V. ヒト表皮細胞の初代培養と継代[15]

外科手術の際ヒトの小児より無菌的に切除した皮膚をメスで表皮部と真皮部とに分け，表皮部を2～3 mm 大でさいの目に細切し，これに0～25％コラゲナーゼ（Sigma Chem. Co., Chicago,

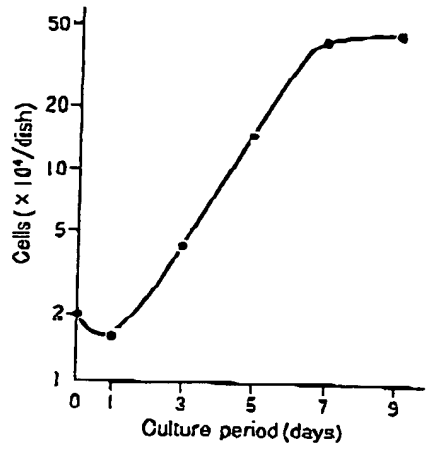

図3 ヒト食道上皮細胞の初代無血清培養の増殖カーブ

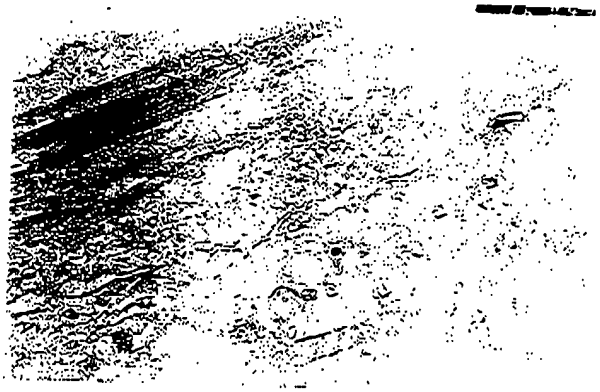

図4 ヒト培養食道上皮細胞の電顕像 “デスモゾームの存在”

IL.）液に60分間放置した後 PBS で2，3回洗滌する。その後トリプシンの低温細胞分散法により単個遊離細胞に分散する。この際トリプシン作用直後0.01％の大豆トリプシン阻止因子（STI）を作用させてトリプシンの作用をとめる方がよい。このようにして分離した細胞をファイブロネクチンでコートしたプラスチックシャーレに接種する。この際使用する培地は前述の RITC 80-7 培地のカルシウム濃度を1/10に減量したものに，牛アルブミン，ハイドロコーチゾンおよび上記のウシの脳抽出物を加えたものを用いた。この培地に生育した細胞は4～5代継代培養が可能で，その PDL は17～19 PDL に達した。勿論培養された細胞の細胞質内には多量のケラチンが含まれていることが螢光抗体染色で証明された（図5参照）。

図 5 ヒト培養食道上皮細胞質内のケラチン（螢光抗体染色）

図 6 ヒト培養胆のう上皮細胞質内のケラチン（螢光抗体染色）

VI. ヒトの胆嚢上皮細胞の初代培養と継代[16]

胆石症の摘出手術材料より得られた胆嚢組織は氷冷，Ca(−)で，0.5mM EGTA 添加の Hanks 液内に保持され，手術摘出後5時間以内に細胞分離に供された。まず胆嚢組織の粘膜側を上にしてペトリー皿内に置き，これに0.025%トリプシン（二回再結晶，Sigma Chemical 社製）液（0.02% EDTA を含む）を加えて細胞分離分散を行った。この際分離細胞を解離するため約15分ごとにピペッティング操作を行った。45分の操作後，得られた細胞懸濁液は遠心して集められた後，PBS で2回ほど洗滌された。洗滌された遊離細胞は径35 mm のペトリー皿には cm^2 当り 2×10^5個，径60mm の皿には 1.5×10^5 個の割合に接種された。

この培養細胞は食道上皮細胞の場合と同様に7%の酸素濃度の低酸素分圧下で incubate (incubate) される必要があった。また用いられた培地は前述の RITC 80-7 培地にヒト二倍体線維芽細胞の conditioned medium を加えたもので，線維芽細胞は培養に用いられた胆嚢組織より得られたものが最も有効であった。この胆嚢上皮細胞は10数 PDL，2～3回継代が可能であった。勿論この細胞の細胞質内にはサイトケラチンの存在が証明され，上皮性であることが確かめられた（図6参照）。

VII. ヒト血管内皮細胞の初代培養と継代

ヒトの血管内皮細胞の培養に関しては Folkman らのグループおよび Maciag らのグループによりすでに行われてきた。彼らは一般に血清添加培地を用い，これに EGF などの通常の成長因子を添加するのみならず，さらに血管内皮細胞に特異的に有効な因子を加えている。このことはウシやブタの血管内皮細胞の培養の場合はまったく不要なので，ヒト内皮細胞の培養と本質的に異なるところである。この特異的に必要な因子として Folkman らは培養がん細胞（主として間葉系腫瘍細胞）の conditioned medium を用いており，この有効因子ががん細胞由来であるので当初 Tumor Angiogenesis Factor (TAF) と称した。一方 Maciag らは有効因子をウシの下垂体および脳の視床下部に見いだし，内皮細胞の培養にそれぞれ用いた。私どもは有効因子がヒトの二倍体線維芽細胞の conditioned medium 中に存在することを認め，これを培地に添加してこの細胞の培養に用いている。なお有効因子はヒトの胎児由来の二倍体線維芽細胞の培養にもっとも多く含まれ，成人皮膚由来の線維芽細胞培養にも存在するが含量はやや少なく，またスキス系3T3の conditioned medium 中にも少量同様の活性を示す因子のあることが判明した[13,14]。

この conditiomed medium を基礎培地の1/2量加えた培地にヒトの臍帯静脈より分離された二倍体血管内皮細胞を50から60数 PDL の長期間継代培養することができる。この際用いた臍帯静脈よりの内皮細胞の分離法は Jaffe が記載したものを少し改変したものである。培養された細胞

表1 ヒト血管内皮細胞培養用無血清培地組成

1. RITC 80-7 basal medium modified from MEM
2. EGF+Insulin
3. Cholesterol+Vitamine E
4. HEL conditioned medium
5. coated with collagene

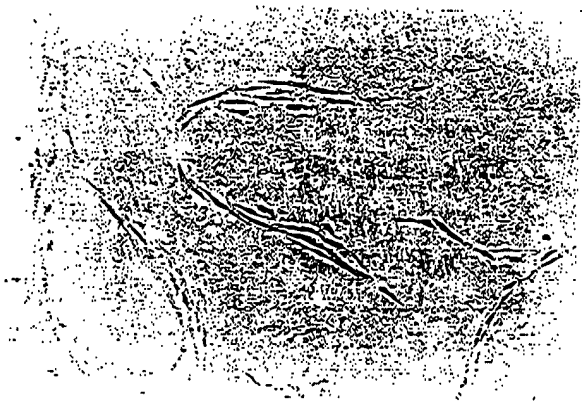

図7 ヒト培養血管内皮細胞の毛細管用構築

はやや三角形をしているが，必ずしも形態的に特異な性質を示すわけではないが，これを血液凝固第八因子に対する抗体で染色すると，すべての培養細胞の細胞質が陽性に染色されるので，これらの細胞が血管内皮細胞であると同定することができる。また私どもは表1に見られる組成の無血清培地を用いて血清添加培地と同様に長期継代培養できるようになった。

ところでこのようにして培養された内皮細胞は生体内で大小の血管の内腔を覆う内皮細胞あるいは毛細血管を形成する内皮細胞の形態とは著しく異なるが，一旦飽和増殖にまで培養したこの細胞をその増殖に必須な上記の conditioned medium の欠如した培地で，2，3日毎日培地交換を繰り返すと，細胞のあるものは剝離して失われるとともにあるものは容器基質上を遊走し，さらに一個一個の形態が著しく細長くなり，毛細血管の net work を思わせる構築を形成し，またあるものは血管の横断面の管腔を思わせる輪状の構築を形成するようになる（図7参照）。

上記の conditioned medium 中に含まれるヒトの血管内皮細胞に対する成長因子の抽出精製を試みた[17]。まずこの有効因子が pH の酸性側では比較的に安定なので，conditioned medium を塩酸で pH 4.0とし遠心して不溶部を取り除く。つぎに上清をアミコン限外ろ過膜 YM-10（米国アミコン社製）を用いて1/10ないし1/20にまで濃縮し，この濃縮された液を遠心後硫酸安門による塩析に供した。すなわち pH 4.5で30%飽和の硫酸安門塩析では溶けるが，70%飽和では沈澱する分画に活性成分の集まることを利用してこれを捕捉した。この分画を集めて透析し，これをヘパリン-セファロース親和クロマトグラフにかけ，その第3，4分画に活性が集中しているので，この分画を溶離して集めた。この分画をさらに DEAE-セファセルのイオン交換クロマトグラフにかけ，その第3分画を集めた。これを TSK ゲル Toyopearl HW-55（東洋曹達社製）によるゲルクロマトグラフにかけ，分子量3万前後の分画を集めて溶出した。この分画は出発の conditioned medium の活性に比べて約820倍精製されており，等電点電気泳動的にはその等電点は5.5附近にあった。

このようなヒトの血管内皮細胞の成長因子に関しては，ハーバート大学 D. J. Strydon ら[18]により，培養がん細胞の conditioned medium から精製された Angiogenine として，そのアミノ酸配列の大様も発表されており，またレプロン社の Maciag らによりウシの脳の視床下部から精製されたものの遺伝子 DNA 塩基配列も発表されている[19]。私どもの精製したものは，いまだその化学構造は明らかになっていないが，その物理化学的性質から Angiogenine よりも Maciag らがウシの脳から抽出したものに近いものだと考えられる。とにかく私どもはヒトの血管内皮細胞成長因子は決して一種だけ存在するのではなく，多く分子種が多元的に存在するのではないかと考えている。

上記のようにして精製された私どもの因子約10 mg 以下（計算上）をポリアクリルアマイドゲルに包み込み，これを家兎の眼球角膜内に（メスで角膜の一部を切りポケット形成後）埋め込むと，埋め込み後約2週間で次第に著明な血管形成が角膜内に見られ，埋め込み後約20日では直径2mm 前後の血管4～5本と他にこれより細い血管が多数みられ，25日頃よりこの現象は次第に消退する。

図 8 若者分離の ECGF の家兎角膜内注入による血管新生像（ポリアクリルアマイドゲルとともに注入）

図 9 図8の対照像（ECGF（－）でポリアクリルアマイドのみ注入）

一方対照として他方の眼球の角膜にポリアクリルアマイドゲルのみを埋め込んだ場合には，その眼球の結膜に充血（injection）は見られたが，角膜部には血管形成は全然見られなかった。いうまでもなく眼球角膜には元来血管は全然存在しない。したがってこの因子埋め込みによって生じた血管は眼球の周辺部の微小な血管から角膜部に延びて拡大したものである。また角膜部に新生した血管の組織学的切片標本を光顕下に観察すると各血管には大きな管腔が形成されているにもかかわらず内皮細胞のみで形成されており，血管平滑筋，血管中膜，外膜は存在せず，丁度脳の静脈洞または毛細血管の構造をとったものであった。上記の家兎を用いた研究はすべて東北大学医学部眼科学教室との共同研究である（図8，9）。

上記のヒトの正常血管内皮細胞の長期培養の研究はこの細胞がいつでも容易に長期に培養できるようになったことにつきるのであるが，たとえば血管外科の領域で細い人工血管を体内で使用する場合に，その内腔で血液が凝固して血栓を形成する危険が大きいが，人工血管の内腔に培養した血管内皮細胞を用いて lining（内張り）を施して，血栓形成を防止することを常法化できる可能性が開けてきたといえるであろう。また眼科領域等では血管の過形成を主徴とする種々の疾患があるが，実験動物に上記精製成長因子を接種して，血管を実験的に形成することができるので，このような疾患の実験的治療への道が開かれたことになる。

一方この精製成長因子の医薬としての可能性を考えると，まず第一に考えられるのはその病変局所への適用であろう。たとえば皮膚の火傷ないし熱傷等の創傷の回復期における肉芽組織形成の際の血管形成不全によるケロイド形成の予防剤として局所塗布等の適用は十分考えられる。閉塞性内動脈炎等による循環障害に基づく栓塞形成局所に，この因子を接種することにより局所の血管形成を促すことが可能となろう。

さらに私どもは上皮性腫瘍の間質（正常線維芽）細胞と腫瘍細胞の増殖との相関関係に注目している。この問題に関しては古くから Folkman らは腫瘍細胞自身が腫瘍血管に対する成長因子を産生しているとして，その因子を前述のように「腫瘍造血管因子（TAF）」と名づけて，腫瘍腫瘤の抽出液または（多くは）間葉性の培養腫瘍細胞の conditioned medium の血管内皮細胞に対する増殖促進作用を報告している。しかし彼らの用いた腫瘍の腫瘤には勿論間質細胞が多量に含まれており，彼らの使用した培養腫瘍細胞の多くは間葉性のものである。私どもは彼らの主張するように，上皮性の腫瘍においても腫瘍細胞が直接内皮細胞成長因子すなわち TAF を産生して，腫瘍血管の形成を助ける可能性をまったく否定するものではないが，それよりもむしろ腫瘍腫瘤における間質細胞が成長因子を産生して腫瘍血管の形成を促進し，その結果腫瘍細胞に対する栄養および酸素の補給を旺盛にして腫瘍細胞の増殖を促し，増殖を促された腫瘍細胞は間質細胞に対する成長因子を産生する（このことは関してにすでに多くの

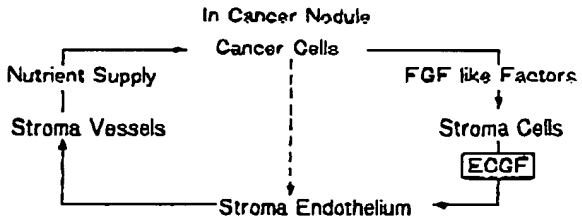

図10 上皮性腫瘍の腫瘤における腫瘍細胞，間質細胞，間質血管増殖における相互関係のシェーマ

報告がある）という一種のサイクリックの相関関係が成立すると考えている。この関係を証明するため，私どもはヒトの食道がんの間質より得られた線維芽細胞を培養して，その conditioned medium がヒトの血管内皮細胞の増殖を強力に促進することを明らかにした。このように腫瘍腫瘤において間質細胞と腫瘍細胞との間に腫瘍血管を介して一種の循環関係のあることを正式に仮設として主張するには，腫瘍腫瘤の無限の増殖膨張を阻止する腫瘍壊死の現象と壊死因子の解明等が一方においてなされなければならないことはいうまでもない（図10参照）。

おわりに

以上ヒトの上皮性細胞や血管内皮細胞等分化機能をもった細胞の初代培養とその長期継代培養に関して，私どもがこの十数年来行ってきた研究を概説的に述べてきた。この中で最も重要なことはいうまでもなく培養に供する生体材料から（無菌的に）目的とする分化細胞を（線維芽細胞等の）他の細胞よりも高い割合で単個細胞に分離分散することである。しかしいうべくして必ずしも実際に可能なことではない。最も問題となるのは（培養に供される）材料より分離された単個細胞の集団の中に混在する線維芽細胞の存在である。一般に線維芽細胞の方が上皮細胞等の分化細胞に比べてはるかに初代培養されやすく，線維芽細胞の優勢増殖に圧倒され，目的とする分化細胞が結果的に培養できないことを私どもはしばしば経験してきた。このような場合に5～10%の血清の添加された培地を用いると，（恐らく血清中に含まれている PDGF の作用によって）目的としない線維芽細胞の方が選択的に優勢増殖して，上記のように目的細胞の培養に失敗することになる。したがって繰り返していうことになるが，分化細胞の初代培養には血清添加培地を用いるにしても，添加濃度は2%以下にすべきである。

このようなことは最近無血清培地や細胞成長因子に関する知見が急速に増加したことによるものではあるが，どの分化細胞でも常法的に初代培養を行うことはいまだ可能ではない。われわれはどの細胞でも自由に初代培養できる手技の確立を目ざして一歩一歩努力すべきであろう。

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Summary

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PRIMARY CULTURES OF VARIOUS DIFFERENTIATED HUMAN CELLS AND THEIR TRANSFER

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From our long experience with primary cultures of human differentiated cells, we have been able to come to the following conclusions. (1) The culture media routinely employed have been developed using established cell lines but not primary cells as the growth marker. Therefore the culture media for primary cultures should be modified from those routinely employed. In addition the concentrated supplementation of serum to basal media dose not contribute to the growth of primary differentiated cells, and in fact on the contrary, is advan tageous for the growth of non-target fibroblasts which would hamper the growth of the target cells. (2) A hypoxic culture environment is more favorable for primary cultures especially when smaller cell numbers are used as the inoculum. The primary culture cells are more fragile in comparison with established cell lines. Therefore, the low-temperature cell dioperaion procedure is recommended with the use of diluted crystalline trypsin saline.

On the basis of the above findings, we have successfully carried out primary and transfer cultures of human esophageal and gall bladder epithelial cells, skin keratinocytes and endothelial cells. Their epithelial mature was proved by the presence of keratin in their cytoplasm, and the phenotype of endothelial cells was evident from the presense of Factor Ⅷ-related antigen in their cytoplasm. Culture of endothelial cells requires the supplementation of a specific growth factor which we have utilized and isolated from conditioned medium of human diploid fibroblasts. Tiny amounts of the factor were shown to, induce the vascularization of rabbit eye cornea.

BIOTECHNOLOGY:

Claim of Human-Cow Embryo Greeted With Skepticism

Eliot Marshall*

A small, privately held company in Worcester, Massachusetts--Advanced Cell Technology Inc.--startled the scientific world last week by announcing that it had fused human DNA with a cow's egg to create a new type of human cell. Company leaders say that a colony of these fused cells--created in 1996, kept alive for 2 weeks, and discarded--looked like a cluster of human embryo cells. On this basis, the company declared that it had "successfully developed a method for producing primitive human embryonic stem cells."

The claim, announced in a front-page news story in *The New York Times* on 12 November, came just 6 days after two groups of researchers reported in *Science* and the *Proceedings of the National Academy of Sciences* that they had used traditional techniques to culture human embryonic stem cells--"undifferentiated" cells that have the potential to grow into any cell type (*Science*, 6 November, pp. 1014 and 1145). It added to the concerns already raised among ethicists and government officials. On 14 November, President Clinton sent a letter to Harold Shapiro, chair of the National Bioethics Advisory Commission (NBAC), saying he is "deeply troubled" by news of the "mingling of human and nonhuman species." The president asked NBAC to give him "as soon as possible ... a thorough review" of the medical and ethical considerations of attempts to develop human stem cells. And a Senate committee may review the company's claim at a hearing on stem cell technology planned for 1 December.

Scientists, however, were startled for another reason: They were amazed that Advanced Cell Technology (ACT) broadcast its claim so widely with so little evidence to support it. Some were puzzled that the company had tried to fuse human DNA and cow eggs without first publishing data on the fusion of DNA and eggs of experimental animals. Many doubted that ACT's scientists had created viable human embryonic stem cells. And most were left wondering why the company chose to go public now with this old experiment.

The company had inserted DNA from adult human cells into cow's eggs using a nuclear transfer technique similar to the one used to clone Dolly, the first mammal cloned from an adult cell. ACT's top researcher and co-founder--developmental biologist James Robl of the University of Massachusetts, Amherst --says an early version of the experiment was performed in his UMass lab "around 1990." A student carrying out nuclear DNA transfer in rabbits had run out of donor cells, Robl recalls, and, almost as a lark, took cheek cells from a technician and transferred their DNA into rabbit oocytes. "I didn't even know about it," Robl says. To everyone's surprise, the cells began to divide and look like embryos. "I got very nervous" on learning about it, Robl says, and shut down the experiment.

Robl and his former postdoc Jose Cibelli, now a staffer at ACT, returned to this line of experimentation in 1995 to '96, when they were working with cow embryos on other projects. They remembered that the human DNA-animal oocyte combination worked before, and "we thought, 'Maybe we can get a cell line' " this way.

Cibelli transferred nuclear DNA [redacted]4 of his own cheek cells and 18 lym[redacted]ocyte cells into cow oocytes from which the nuclei had been removed. Six colonies grew through four divisions, according to Cibelli, but only one cheek cell colony grew beyond that stage--reaching 16 to 400 cells. Robl says they didn't follow up on the work because "we had about 15 other things we were doing," and developing human stem cells was not at the top of the list. But the university did file for a **patent** on the technique, granting an exclusive license to ACT.

Robl concedes that the experiment did not yield publishable data. He says he classified the cells as human stem cells based on his experience of "look[ing] at hundreds and hundreds" of cell colonies. But Robl offered no other data to support this conclusion.

Other researchers agree that the cells may have had human qualities, because they continued to divide after the cow's nuclear DNA had been replaced with human DNA. But Robl and Cibelli didn't do any of the tests normally done to show that these cells were human or that they were stem cells, such as looking for expression of human proteins or growth of specialized tissues. James Thomson of the University of Wisconsin, Madison, lead author of the *Science* paper, says that ACT's cells "meet none of the criteria" for embryonic stem cells. And Gary Anderson of the University of California, Davis, who has isolated a line of embryonic pig cells, comments: "Just because someone says they're embryonic stem cells doesn't mean they are."

A few researchers--including Robert Wall, a geneticist at the U.S. Department of Agriculture in Beltsville, Maryland--were willing to suspend their disbelief, however, if only because they respect Robl. He is "a top-notch, very solid scientist," says Wall, who adds that anyone who has examined a large number of embryonic cells can distinguish real ones from impostors.

But others are less charitable. "This may be another Dr. Seed episode," says Brigid Hogan, an embryologist at Vanderbilt University in Nashville, Tennessee, referring to Chicago physicist Richard Seed, who caused a furor early this year when he announced that he planned to clone humans. Although Seed didn't have the means to carry out his project, Congress quickly drafted a criminal ban on many types of cloning research. Congress set that debate aside last spring but indicated it might take it up again later (*Science*, 16 January, p. 315 and 20 February, p. 1123). Hogan, a member of a 1994 National Institutes of Health (NIH) panel that proposed guidelines for human embryo research, agrees that "it's theoretically possible" to do what ACT claims to have done. But the company's announcement reminds her of the Seed case because "it smells to me of sensationalism" and seems "likely to inflame an uninformed debate."

Why did ACT publicize this experiment now? Some observers think the company wanted to ride the PR bandwagon created by the 6 November announcements by the labs that had isolated human embryonic stem cells using more traditional culture techniques. One group, led by developmental geneticist John Gearhart at The Johns Hopkins University, extracted primordial germ line cells from fetal tissue and kept them growing through 20 passages (transfers from one plate to another) for more than 9 months. The other group, led by Thomson at the University of Wisconsin, established a culture of stem cells derived from early human embryos. Thomson, whose cell line has survived 32 passages over 8 months, published molecular data suggesting that the cells may continue dividing "indefinitely."

Michael West, president and chief executive officer of ACT since October, says it is "pure coincidence" that ACT's news came out within a week of these announcements. West--noting that ACT won't benefit immediately, for it doesn't sell public stock--says that after becoming ACT's CEO last month, "I learned about the work that had been done in 1996 ... and I wanted to develop this technology." But he says he "didn't feel comfortable" moving ahead with nuclear DNA transfer experiments without getting a reading on how future U.S. laws and regulations might affect the field. "So I decided, 'Let's talk about the preliminary results,' " says West. "Let's get NBAC to help clear the air."

West notes that some information on ACT's mixing of human and cow cells was already public. In February, the World Intellectual Property Organization in Geneva had published Robl's application for a **patent** on "Embryonic or Stem-like Cell Lines Produced by Cross Species Nuclear Transplantation" (WO 98/07841). It

describes the Robl-Cibelli experi[redacted]f 1996 and stakes broad claims to s[redacted] cell technology based on transferring human or animal DNA into an animal oocyte. After being approached by the staff of CBS's news show *48 Hours*, West says, he arranged to discuss the research in exclusive but simultaneous releases to *The New York Times* and CBS. The CBS report aired on 12 November.

Robl confirms it was West, and not the scientific staff at ACT, who initiated the announcements. "I wouldn't have had the guts to do it," Robl says, although he agrees it is important to debate ethical concerns that might impede the technology.

These ethical concerns may get an airing next month. Senator Arlen Specter (R-PA), chair of the appropriations subcommittee that approves the budget for NIH, is planning a hearing on 1 December. There, NIH director Harold Varmus and developers of new human cell technologies are expected to testify about federal restrictions on the use of embryonic and fetal tissue and their impact on biomedical research. That discussion may now be expanded to include questions about ACT's single experiment.

With reporting by Elizabeth Pennisi.

Volume 282, Number 5393 Issue of 20 Nov 1998, pp. 1390 - 1391

Reuters Medical News for the Professional

Dolly And Other Cloned Sheep Contain Mitochondrial DNA Exclusively From Recipient Oocytes

WESTPORT, Sep 01 (Reuters Health) - Sheep cloned from adult somatic cells contain mitochondrial DNA (mtDNA) that derives exclusively from enucleated recipient oocytes used in the nuclear transfer process, according to a report in the September issue of *Nature Genetics*.

Dr. Eric Schon from Columbia University College of Physicians and Surgeons in New York and colleagues there and at the Roslin Institute in Edinburgh, Scotland, explain that during mammalian cloning, donor somatic cells are fused with recipient enucleated oocytes by a process called electroporation. During electroporation, both nuclear DNA and mtDNA are transferred from donor to recipient. Consequently, the resulting cloned animal would be expected to contain both donor and recipient mtDNA.

Dr. Schon's team analyzed the highly variable D-loop region of mtDNA from donor somatic cells, recipient oocytes, and cloned sheep using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis.

"On RFLP analysis, all somatic donor cell and representative oocyte-recipient [representative Scottish Blackface sheep oocyte] samples displayed the predicted patterns," the results indicate.

RFLP analysis of the same mtDNA region from ten cloned sheep "...showed that all ten were homoplasmic for the mitochondrial genotype of the [Scottish Blackface sheep] recipient oocyte in all tissues examined, with no evidence for the presence of donor mitochondria-derived mtDNA," the investigators report. "This indicates that the mtDNA genotypes of the nuclear transfer-derived sheep do not match those of their nuclear donors."

"The finding of mtDNA homoplasmy in all ten nuclear transfer-derived sheep was unexpected," the authors write. "The mtDNA composition of each fetal sheep derived by nuclear transfer

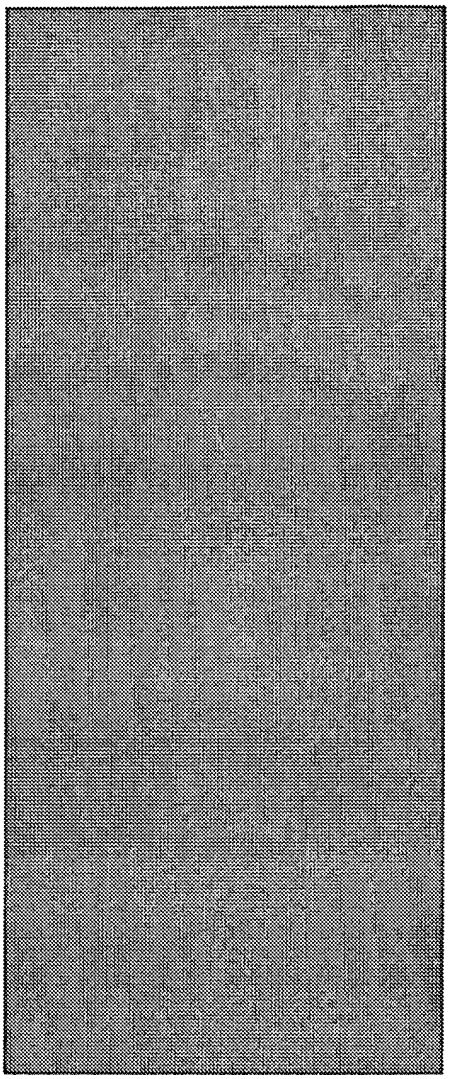
should reflect that of the original electrofused donor/recipient cell...in which case we should have observed 2-5% of donor-derived heteroplasmic mtDNA in the samples."

Several explanations may account for these findings. The authors "...favor a scenario in which an active mechanism operates to destroy the donor mitochondria in the recipient ooplasm, similar to what is thought to happen to sperm-derived mitochondria in fertilized ova in...normal human reproduction."

The results suggest possible treatment strategies for such human mitochondrial diseases as mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes, and maternally inherited Leigh syndrome, the investigators write.

Such disorders might be corrected, for example, by "...nuclear transfer involving a somatic or germline cell from a woman harboring a pathogenic mtDNA mutation (but normal nuclear DNA) and a recipient enucleated oocyte (containing normal cytoplasm). If the experience with cloned sheep is any guide," the authors conclude, "we would predict that the human mitochondrial genotype will be determined by the recipient ooplasm."

Nature Genetics 1999;23:90-93.



Cell Prolif. 1996, **29**, 309–314

Growth factors and apoptosis

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(Invited presentation at ESGCP September meeting in Uppsala)

Abstract. Apoptosis is nowadays recognized as an important mechanism by which cells can be eliminated from the organism. In particular its role in tissue modelling during embryogenesis has been highlighted. The human teratoma cell line Tera 2, which in several respects acts as a human embryonic stem cell, can be induced to undergo apoptosis by reducing the serum content of the tissue culture medium. We report here that this process can be reversed by replacing serum with the heparin-binding growth factors, acidic FGF and basic FGF. In contrast, neither of the mammalian transforming growth factors (TGF-β1-3) managed to exert any effect on growth or apoptosis in Tera 2 cells.

More than 20 years ago, Sir Alistair Currie and his co-workers proposed the existence of two different types of cell death (Kerr *et al.* 1972). Firstly, necrosis is caused by physical damage to groups of cells (e.g. by hypoxia or chemical toxins) and provokes an inflammatory response. Secondly, apoptosis occurs in individual cells, often surrounded by unaffected neighbours. Apoptosis is characterized by a striking series of morphological transformations which eventually disassembles into apoptotic bodies which are membrane-enclosed vesicles. Apoptosis is an active process requiring ATP as well as a functional machinery for *de novo* protein synthesis. Moreover, it does not provoke an inflammatory response. Rather, the apoptotic bodies are engulfed by local macrophages. It was therefore concluded that apoptosis represented a new form of cell death.

We have recently drawn attention to the human embryonal carcinoma cell line Tera 2 as a useful model system for studies of apoptosis (Granerus *et al.* 1994). Tera 2 cells can be readily induced to differentiate *in vitro* by exposure to retinoic acid (Thompson *et al.* 1984). As a xenograft, it gives rise to tumours containing cells originating from different germ layers. It has therefore been proposed that Tera 2 cells are the best available substitute for a true human embryonic stem cell (Schofield & Engström 1992). Tera 2 cells were recently shown to undergo morphologically confirmed apoptosis after serum withdrawal (Granerus *et al.* 1994, Granerus *et al.* 1995a, b). Moreover, the phenomenon was confirmed by the appearance of nucleosome ladders (Granerus *et al.* 1995a, b). The purpose of the current study was to examine the effects of heparin-binding growth factors and transforming growth factors on proliferation and apoptosis in Tera 2 cells.

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MATERIALS AND METHODS

Cell culture

The human teratoma derived cell line was established and maintained as described in Thompson *et al.* (1984). Serum-free culture experiments were conducted as described in Engström *et al.* (1985), Engström (1986), Biddle *et al.* (1988).

Growth factors and tissue culture materials

The growth factors acidic FGF (aFGF), basic FGF (bFGF) and the transforming growth factors beta 1–3 (TGF β1–3) were purchased from British Biotechnology, Oxford, UK. Transferrin was acquired from Boehringer Mannheim (Sweden) and preloaded with iron according to the manufacturers instructions. α-MEM, trypsin and tissue culture plastic were obtained from KEBO, Sweden.

Assessment of cell proliferation and apoptosis

The proliferation of Tera 2 cells over a 24-h or 5-day period was monitored by counting cell numbers and pulse labelling with tritiated thymidine followed by autoradiography, as described by Biddle *et al.* (1988). The assessment of intact *v.* apoptotic cells was performed by acridine orange staining and fluorescence microscopy as described by Granerus *et al.* (1994). That nuclear fragmentation corresponded to nucleosome laddering was confirmed according to Granerus *et al.* (1994).

RESULTS

Figure 1 shows the effect of growth factor addition on Tera 2 cells over a 24-h period. It was found that 10 ng of acidic or basic FGF per ml of medium exerted an observable effect on cell numbers. In contrast, neither 10 ng TGF-β1, TGF-β2 or TGF-β3 per ml of medium exerted any effect on Tera 2 cell numbers over a 24-h period. Moreover, even when we added the three TGF-βs in concentrations ranging from 0.1 to 100 ng/ml alone or in combination, in no case did we achieve any effects on Tera 2 cell numbers (data not shown).

Table 1 shows the proportion of Tera 2 cells traversing S phase. Cell cultures were exposed to serum-free medium with or without supplementation of 10 ng bFGF, aFGF, TGF-β1, TGF-β2 or TGF-β3 per ml. The proportion of cells undergoing DNA replication at a given moment was assayed by pulse-labelling with tritiated thymidine 24 or 48 h after medium change and subsequent autoradiography. The results clearly indicate that the percentage of [^{3}H]-thymidine labelled cells was virtually unchanged when Tera 2 cells were deprived of serum. The addition of heparin-binding growth factors or transforming growth factors did not significantly alter the proportion of S phase cells.

Figure 2 shows the growth of Tera 2 cells over a 5-day period under different conditions. Whereas cells growing in 10% serum undergo exponential growth, cell cultures in a serum-free medium are maintained at a steady-state level. When 10 ng/ml of aFGF or bFGF was added to serum-free medium, multiplication of Tera 2 cells was supported over a 5-day period. In contrast, when 10 ng of TGF-β1, 2 or 3 was added, no effect on cell numbers could be observed. Nor was any effect on cell proliferation observed when higher or lower concentrations (0.01–100 ng/ml) of the three transforming growth factors—alone or in combination—was added (data not shown).

Figure 3 shows the effect of growth-factor supplementation to a serum-free medium on survival in Tera 2 cells. This was measured by staining Tera 2 cells with acridine orange and

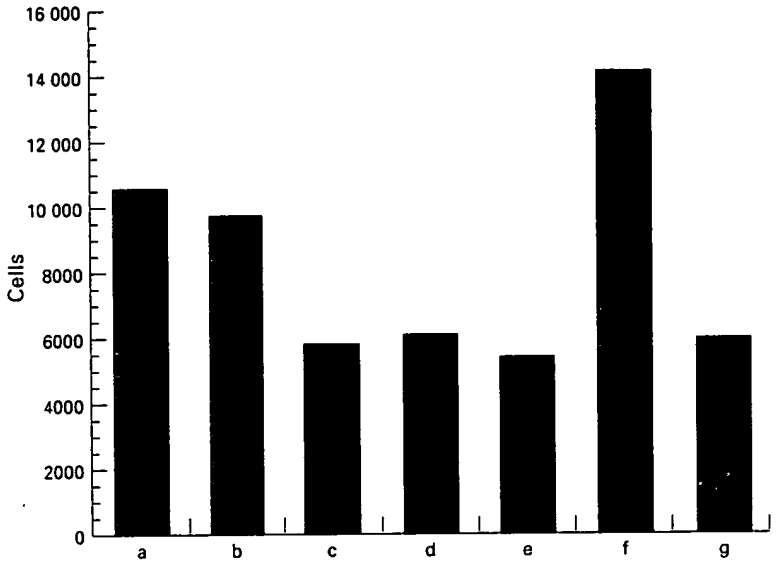

Figure 1. Effect of growth factors on the proliferation of Tera 2 cells in serum-free medium. Cells were seeded onto 35-mm dishes in 10% serum. After 24 h serum was removed and the cultures exposed to serum-free medium containing transferrin and (a) 10 ng aFGF/ml, (b) 10 ng bFGF/ml, (c) 10 ng TGF-β1/ml, (d) 10 ng TGF-β2/ml or (e) 10 ng TGF-β3/ml, (f) 10% serum, and (g) serum-free medium. After 24 h the cells were trypsinized off the dishes and counted in a Coulter counter. The experiment was repeated and the cells counted in triplicate.

judging the proportion of intact *v.* fragmented nuclei with a fluorescent microscope. It was found that serum withdrawal reduced the number of intact nuclei from 90 to 35% over the first 24 h. Between the 2nd and 5th days the proportion of intact nuclei stabilized at approximately 40%. It was found that addition of 10 ng/ml of aFGF or bFGF resulted in a substantially higher proportion of intact nuclei over the entire 5-day period. In contrast, supplementation with 10 ng TGF-β1, 2 or 3 did not in any way alter the numbers of intact

Table 1. Tera 2 cells growing on glass coverslips were exposed to media as specified for 24 or 48 h. During the last hour prior to fixation, the cultures were exposed to 370 kBq tritiated thymidine per ml medium. After fixation the slides were washed in cold trichloroacetic acid and subjected to autoradiography. After development, the slides were counted under a light microscope. The figures represent a duplicate experiment where at least 500 cells per experimental situation were counted

	% Labelled cells	
Medium	Labelling 23–24 h	Interval 47–48 h
10% serum	35.1	35.7
Serum-free medium (SFM)	36.6	31.5
SFM + 10 ng aFGF/ml	32.7	31.0
SFM + 10 ng bFGF/ml	35.0	34.1
SFM + 10 ng TGF-β1/ml	33.8	34.8
SFM + 10 ng TGF-β2/ml	33.8	37.9
SFM + 10 ng TGF-β3/ml	35.1	30.6

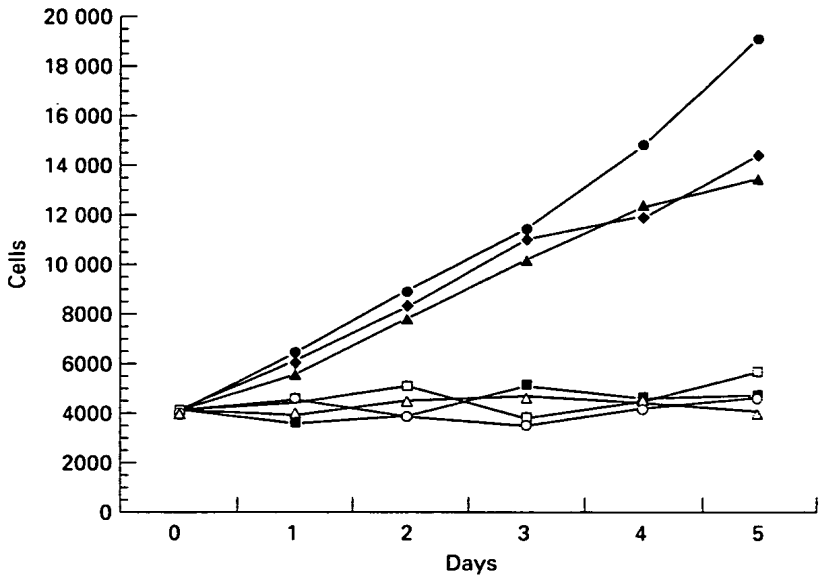

Figure 2. The effect of growth factors on the proliferation of Tera 2 cells. Cells were seeded onto 35-mm dishes in 10% serum. After 24 h (day 0) the serum-containing medium was removed and the cells exposed to serum-free medium containing transferrin only ■, 10% serum ●, 10 ng aFGF/ml ▲, 10 ng bFGF/ml ◆, 10 ng TGF-β1/ml ○, 10 ng TGF-β2/ml □, or 10ng TGF-β3/ml △. Dishes were trypsinized and counted in triplicate daily throughout a 5-day assay period. The figure represents the means of three different experiments.

nuclei in serum-free cultures. Nor did we observe any effect of any other TGF-β concentration (data not shown). When TGF-β1, 2 or 3 was added together with 10 ng/ml of aFGF or bFGF, there was no enhancing or suppressing effect on cell numbers or the proportion of cells with intact nuclei. Neither of the TGF-βs altered the growth characteristics of Tera 2 cells growing in 10% serum (data not shown).

DISCUSSION

Apoptosis is the highly conserved mechanism by which cells are capable of committing suicide. Several independent lines of reasoning have fostered the argument for a link between the traditional eukaryote cell cycle and apoptosis (Evan *et al.* 1995). We know, for instance, that developing tissues exhibit an appreciable degree of apoptosis and that tumours that have lost control of their proliferation have often undergone anti-apoptotic mutations. One of the interesting links between life and death on a cellular level are the polypeptide growth factors. It has been convincingly shown that, in certain cell lines that are unable to enter G_0, after exposure to suboptimal growth conditions only one option is possible, to cycle or to die (Evan *et al.* 1992, Harrington *et al.* 1994, Granerus *et al.* 1994, 1995a, b). The decision to continue to cycle, i.e. to avoid apoptosis, is believed to depend on the presence of growth factors such as platelet-derived growth factor (PDGF) (Harrington *et al.* 1994), leukemia inhibitory factor (Granerus *et al.* 1994) or the insulin-like growth factors, IGF I or IGF II (Harrington *et al.* 1994, Granerus *et al.* 1995a, b).

This study has shown that the heparin-binding growth factors, acidic FGF (aFGF) and basic FGF (bFGF), play a similar role in counteracting apoptosis in a human teratoma cell

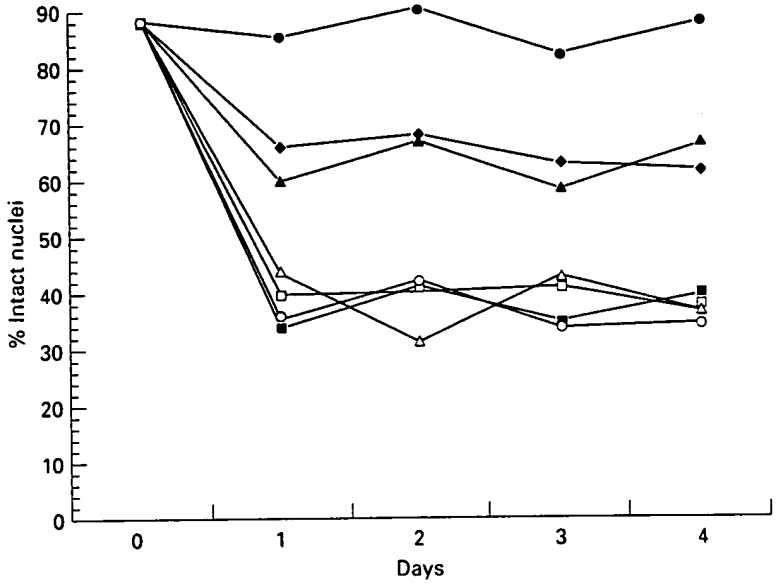

Figure 3. Effect of growth factors on apoptosis in Tera 2 cells in serum-free medium. Cells were seeded onto gelatin-treated glass coverslips in Petri dishes in 10% serum. After 24 h the serum containing medium was removed and the cells exposed to serum-free medium containing transferrin only ■, 10% serum ●, 10 ng aFGF/ml ◆, 10 ng bFGF/ml ▲, 10 ng TGF-β1/ml ○, 10 ng TGF-β2/ml □, or 10 ng TGF-β3/ml △. Glass slides were removed daily and stained with acridine orange and examined by fluorescence microscopy (435 nm). The percentage intact nuclei was recorded by counting at least 200 cells per glass slide. The figure represents the means of three different experiments.

line (Tera 2) which is characterized by its dependence on serum for survival. The heparin-binding growth factors are of particular interest since both aFGF and bFGF have been shown to exert different biological effects in the Tera 2 cell line. When low concentrations of bFGF are added, Tera 2 cells are stimulated to proliferate. When higher concentrations are added, the cells cease to proliferate and increase their locomotion activities (Schofield *et al.* 1992, Granerus *et al.* 1993). This study has demonstrated that, by using a combination of morphological examination of single cells, autoradiography and determination of cell numbers, the effect on Tera 2 cell proliferation is executed by decreasing the proportion of cells that undergo apoptosis. This finding is in line with previous results demonstrating a distinct anti-apoptotic effect by the insulin-like growth factors IGF I and IGF II (Granerus *et al.* 1995a, b).

ACKNOWLEDGEMENTS

This study was generously supported by grants from Cancerfonden and Barncancerfonden.

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MOLECULAR REPRODUCTION AND DEVELOPMENT 38:264–267 (1994)

Nuclear Transplantation by Microinjection of Inner Cell Mass and Granulosa Cell Nuclei

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ABSTRACT The developmental potential of bovine inner cell mass (ICM) and somatic differentiated (granulosa cell) nuclei was investigated using nuclear transplantation. ICM blastomeres were isolated after immunosurgery of day 7 in vitro produced blastocysts and cumulus granulosa cells recovered from in vitro matured oocytes. Nuclear transplantation was carried out by microinjection of the lysed donor cells into enucleated mature oocytes. Oocytes were activated by three 0.2 $kVcm^{-1}$/20 μs pulses in mannitol containing 100 μM Ca^{2+}, with each pulse 22 min apart. Embryos were cultured in vitro for 7 days and blastocysts were transferred into recipients. ICM and granulosa cell donor nuclei directed 7% (20/304) and 9% (19/213) development to blastocysts, respectively. Fifteen blastocysts from ICM donors resulted in four pregnancies (27%) and two births. No pregnancy was detected with granulosa cell donors. The results illustrate the totipotency of ICM nuclei and indicate that granulosa cell nuclei promote preimplantation development of nuclear transplant embryos.

Key Words: Inner cell mass, Granulosa cell, Nuclear transfer, Embryo, Bovine

INTRODUCTION

Amphibian embryonic nuclei microinjected into oocytes direct development to normal adults, whereas nuclei from many differentiated tissues do not. Larvae (Briggs and King, 1952) and fertile adults (Gurdon, 1962) were produced from transplanted embryonic and larval nuclei. However, with differentiated donor nuclei, development proceeds to tadpole stages, but not to adults (Gurdon et al., 1975; DiBerardino et al., 1986). Similar development was obtained with germ cell nuclei from larval and adult frogs (DiBerardino and Hoffner, 1971). These results indicate that embryonic and larval nuclei promote development to fertile frogs. However, the developmental potential becomes more restricted with differentiated nuclei, and many nuclei from adult tissues direct development only part way (DiBerardino, 1987).

Nuclear transplantations in mammals have also been informative about changes in embryonic nuclei during development. Cleavage- and morula-stage donor nuclei direct development to term (Robl et al., 1992), and inner cell mass (ICM) nuclei promote development to blastocysts (Modlinski, 1981; Tsunoda et al., 1989; Collas and Robl, 1991; Kono et al., 1991) and to term (Illmensee and Hoppe, 1981; Smith and Wilmut, 1989). Trophectoderm (TE) nuclei, however, lead to abortive preimplantation development (Illmensee and Hoppe, 1981; Modlinski, 1981; Collas and Robl, 1991). Furthermore, mouse donor primordial germ cells lead to implantation sites but no development to term (Tsunoda et al., 1989). These studies suggest that as development proceeds, the developmental potential of nuclei becomes restricted.

No reported studies have investigated the developmental potential of bovine ICM and differentiated nuclei. Mouse thymocyte nuclei direct limited development to blastocysts but not to term (Kono et al., 1991), and rabbit granulosa cell donor nuclei allow development to blastocysts (Collas and Robl, unpublished). We report here the development to term and to blastocysts of nuclear transplant bovine embryos from ICM and granulosa cell donor nuclei, respectively.

MATERIALS AND METHODS

Oocyte Maturation and Selection

Bovine oocytes from 1–10 mm follicles were matured in vitro for 22 hr at 39°C in a humidified atmosphere of 5% CO_2 in air (Powell and Barnes, 1992). Maturation medium was TCM 199 (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS), 0.01 U/ml follicle stimulating hormone (FSH) (Nobl, Sioux Center, IA), 0.01 U/ml luteinizing hormone (LH) (Nobl), and 1% penicillin G (10 mg/ml)-streptomycin (25 μg/ml) solution (PS). After maturation, the cumulus was removed by vortexing and oocytes were selected for the presence of the first polar body and cultured in Modified Brinsters Ovum Culture Medium (MBMOC; Moore and Bondioli, 1991) until use.

Received December 24, 1993; accepted February 1, 1994.
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TABLE 1. Efficiency of Nuclear Transplantation by Cell Microinjection

	No. oocytes (% injected)		No. embryos (% cultured)					No. embryos (% transferred)			
Cell type	Inj.[a]	Cult.[a]	2-Cell	8-Cell	Morula	Blast.	ET[a]	Recip.[a]	Ext. cycles[b]	Preg.[a]	Calves
ICM	903	304 (33)	152 (50)	77 (25)	33 (11)	20 (7)	15 (5)	15	?	4 (27)	2
Granulosa	351	213 (61)	131 (62)	79 (37)	29 (14)	19 (9)	19 (9)	13	7	0 (0)	0 (0)

[a]Inj., injected; Cult., cultured; ET, no. of embryos transferred; Recip., no. of recipients.
[b]Ext. cycles, no. of recipients with extended estrous cycle (>30 days) that were detected nonpregnant at day 45 post transfer.

Oocyte Enucleation

Mature oocytes were incubated in the DNA-specific stain, Hoechst 33342, for 15 min at 39°C and placed in 50 μl drops of Tyrode's solution (TL Hepes), containing 5 μg/ml cytochalasin B, under oil. Manipulation was carried out at 200× under phase contrast. Metaphase chromosomes were aspirated and enucleation was assessed by exposing oocytes under UV light for 1 sec. Enucleated oocytes were cultured until blastomere injection.

Isolation of ICM Blastomeres

Inner cell masses were obtained by immunosurgery of zona-free blastocysts. The zona pellucida of day 7 in vitro fertilized embryos was digested for 3 min at 39°C with 0.1% pronase E (Sigma) in phosphate buffered saline (PBS) containing 3 mg/ml bovine serum albumin (BSA). Embryos were washed in PBS containing 20% FCS, and gentle pipetting completed removal of the zona pellucida. Zona-free blastocysts were incubated in MBMOC containing rabbit anti bovine serum (Sigma B-8270; 5:1, vol:vol) for 30 min at 39°C. Embryos were washed twice in PBS and incubated for 10 min at 39°C in 5:1 MBMOC to guinea pig complement (Sigma S-1639). The complement initiated lysis of trophectoderm cells with bound antibodies. The ICM was pipetted in PBS to help remove the lysed trophectoderm. To isolate blastomeres, the ICM was incubated for <2 min in 0.1% pronase E and pipetted with a 40 μm fire-polished bore pipette.

ICM Blastomere Injection

At 27 hpm, ICM blastomeres were placed in a 50 μl drop of TL Hepes under oil. Enucleated oocytes were placed in an adjacent drop of TL Hepes containing 5% sucrose (wt/vol). Oocytes remained in sucrose for ≈1 hr for the injection procedure. The injection pipette had a sharp, beveled 8 μm tip. Blastomeres were lysed by rupturing the plasma membrane with the injection pipette and the lysate was aspirated in the pipette. It was important to keep the lysate as concentrated as possible in the pipette to limit the volume injected into the oocyte cytoplasm. After injection, oocytes were rehydrated in TL Hepes containing 2.5% sucrose for 5 min, then in the absence of sucrose for an additional 5 min prior to culture until activation. Sixteen replicates were performed.

Granulosa Cell Isolation and Injection

Cumulus granulosa cells were used because they have been shown to direct development of nuclear transplant (NT) rabbit embryos to blastocysts (Collas and Robl, unpublished). Also, they are easily isolated from in vitro matured oocytes. At 22 hr post onset of maturation (hpm), a mature oocyte with expanded cumulus was isolated and a portion of the cumulus was separated from the oocyte with a glass needle. The cumulus was cultured in maturation medium until use. At 27 hpm, the cumulus was vigorously pipetted in TL Hepes to isolate individual cells. Cells were placed in a 50 μl drop of TL Hepes and lysed with the injection pipette (4 μm tip) as described. Microinjection was carried out as described and six replicates were performed.

Oocyte Activation

At 30 hpm, manipulated oocytes were incubated for 2 min in 35–37°C 0.27 M mannitol solution containing 100 μM $CaCl_2$, 100 μM $MgCl_2$, and 0.01 mg/ml BSA. They were then placed in a chamber consisting of two electrodes 0.5 mm apart overlaid with 35–37°C mannitol. Oocytes were stimulated with three 0.2 $kVcm^{-1}$/20 μs pulses, each pulse 22 min apart. Oocytes were placed in culture between pulses (Collas et al., 1993).

Embryo Culture and Embryo Transfer

Culture medium was MBMOC and incubator conditions were as above. Embryos were cultured for 3 days in MBMOC and for 4 days with BRL cells (ATCC, 3TA) (Voelkel et al., 1992). Blastocysts were incubated in 37°C CO_2-equilibrated TCM 199 with 10% FCS and shipped for transfer into recipients. Pregnancy detection was done by ultrasonography at 45, 60, and 90 days after transfer.

RESULTS

The efficiency of nuclear transplantation by microinjection of ICM and granulosa cells is presented in Table 1. With ICM donor nuclei, 7% (20/304) of cultured embryos developed to blastocysts. Fifteen blastocysts were transferred into recipient cows and resulted in four

(27%) pregnancies and two births. With granulosa cell donor nuclei, 9% (19/213) of the cultured embryos developed to blastocysts. Blastocysts were transferred into recipients, but no pregnancy was detected by ultrasonography at day 45.

DISCUSSION

This study illustrates the totipotency of ICM nuclei and supports the pluripotency of transplanted differentiated nuclei. Previous limited studies in mammals also reported development to term with ICM donor nuclei (sheep: Smith and Wilmut, 1989; bovine: Keefer, personal communication) and preimplantation development with different somatic cell types. Mouse thymocyte nuclei promote development to blastocysts (Kono et al., 1991), and rabbit NT blastocysts have been produced from cumulus granulosa cell and fetal fibroblast donor nuclei (Collas and Robl, unpublished). Development to blastocysts with granulosa cell and thymocyte donor nuclei suggests that a variety of differentiated mammalian cell types may promote early preimplantation development of NT embryos.

Nuclear transplantations have demonstrated the restriction of the developmental capacity of differentiated somatic nuclei. Larval stages of development were obtained by transplantation of various differentiated cells of embryonic, larval, and adult frogs (DiBerardino, 1987). NT adults, however, resulted from the transfer of embryonic and larval, but not adult, nuclei. In mammals, NT adults have been produced from the transfer of early embryonic cells only (Robl et al., 1992). There is evidence, therefore, for the totipotency of some embryonic and larval nuclei, whereas the developmental potential of adult nuclei is much more restricted.

Restricted developmental potential of nuclei from advanced larvae and adults indicate that changes have occurred in these nuclei that are not easily reversible. These changes are believed to be the result of cellular differentiation (reviewed by Gurdon, 1986; DiBerardino, 1987). If these changes are nuclear, they can be related primarily to DNA replication and RNA transcription. Evidence indicates that chromosomal aberrations caused by incomplete DNA replication may cause restricted development in amphibian nuclear transplants (Gurdon, 1986; DiBerardino, 1987). Incomplete DNA replication may be result from asynchrony in the length of the cell cycle between donor nuclei and recipient oocytes in amphibians (DiBerardino, 1987) and, to some extent, in mammals (Collas et al., 1992; Barnes et al., 1993). Incomplete DNA replication may also be due to changes in transcriptionally active and inactive regions of DNA and their timing of replication. As cells differentiate, many genes become transcriptionally inactive. A failure in development of NT embryos may be due to their inability to re-activate inactive genes. Evidence to support this possibility comes from studies by Briggs and King (1957), in which nuclei derived from specific lineages gave rise to well-developed tissues of the same lineage but not other lineages. Whether this would also occur in mammalian NT embryos cannot be determined to date because of limited embryonic development. Cell differentiation, therefore, is accompanied by stable developmental restrictions that to date cannot be easily reversed by nuclear transplantation.

ACKNOWLEDGMENTS

The authors are grateful to the staff of Trans Ova Genetics (Sioux Center, Iowa) for performing embryo transfers and pregnancy detections.

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and differentiate in an embryo *in vivo*, contributing somatic tissues or germ cells. ES cells offer the same potential advantages for the genetic engineering of farm animals that have been realized in mice, namely the ability to preselect for a desired genetic modification and generate, delete, or directly modify endogenous genetic traits by using gene targeting protocols. In species in which conventional random integration transgenics are difficult to make, the availability of the ES cell route offers the additional advantage of being able to preselect for a desired integration structure or site for a transgene. The existance of such cell lines would greatly enhance our ability to genetically modify farm animals for the purposes of species improvement or protein production.

In the context of the whole organism, embryonic stem cells offer many opportunities because virtually any genetic change can be introduced into ES cells in vitro and established as a mouse *in vivo*. These include the mutational analysis of endogenous genes at a gross or fine level, the directed expression of a transgene by targeting it to the *cis* elements of an endogenous gene, and the delivery of very large transgenes such as those constructed in the context of YAC vectors. At present, we are less restricted by what we can do than by the imprecise understanding of the developmental biology and physiology of what we have done.

Acknowledgments

Work in the authors' laboratory is supported by grants from the NIH, The Cystic Fibrosis Foundation and the Searle Scholars Program/Chicago Community Trust. AB is a Leukemia Society fellow.

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Mitochondrial DNA genotypes in nuclear transfer-derived cloned sheep

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Eukaryotic cells contain two distinct genomes. One is located in the nucleus (nDNA) and is transmitted in a mendelian fashion, whereas the other is located in mitochondria (mtDNA) and is transmitted by maternal inheritance. Cloning of mammals[1–6] typically has been achieved via nuclear transfer, in which a donor somatic cell is fused by electoporation with a recipient enucleated oocyte. During this whole-cell electrofusion, nDNA as well as mtDNA ought to be transferred to the oocyte[7,8]. Thus, the cloned progeny should harbour mtDNAs from both the donor and recipient cytoplasms, resulting in heteroplasmy. Although the confirmation of nuclear transfer has been established using somatic cell-specific nDNA markers, no similar analysis of the mtDNA genotype has been reported. We report here the origin of the mtDNA in Dolly, the first animal cloned from an established adult somatic cell line, and in nine other nuclear transfer-derived sheep generated from fetal cells. The mtDNA of each of the ten nuclear-transfer sheep was derived exclusively from recipient enucleated oocytes, with no detectable contribution from the respective somatic donor cells. Thus, although these ten sheep are authentic nuclear clones, they are in fact genetic chimaeras, containing somatic cell-derived nuclear DNA but oocyte-derived mtDNA.

Several aspects of mitochondrial genetics are relevant to the understanding of mitochondrial transmission during nuclear transfer. First, there are approximately 100,000 copies of mtDNA (a double-stranded DNA circle of ~16–17 kb) in a mammalian oocyte[9,10], but only a few thousand copies in a typical mammalian somatic cell[11]. Second, there is no mtDNA replication until the blastocyst (~100-cell) stage of embryogenesis[9]; thus, each of the 20 cells of the inner cell mass that ultimately develop

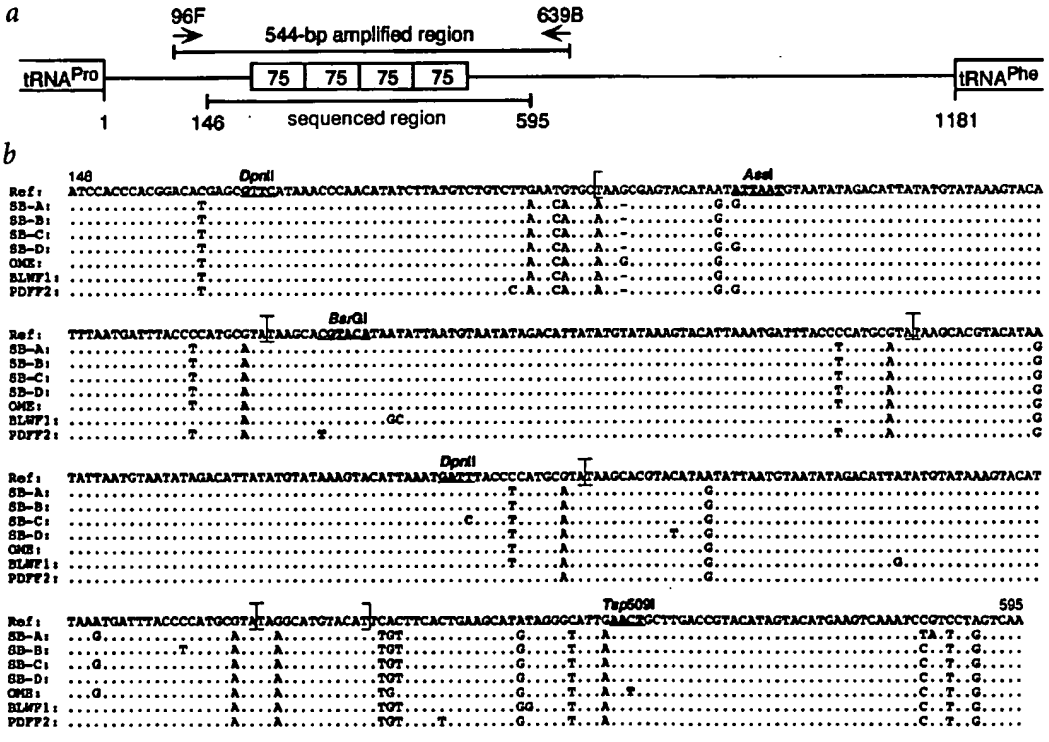

Fig. 1 The sheep D-loop region. *a*, Map of the region, flanked by the genes encoding $tRNA^{Pro}$ and $tRNA^{Phe}$, showing four 75-bp tandem repeats (boxed), the PCR-amplified region (primers indicated above arrows) and the sequenced region. Nucleotide numbering (below map and in *b*) is according to ref. 15. *b*, DNA sequence of the 544-bp PCR-amplified region. Polymorphisms compared with the published reference sequence[15] are shown. The 75-bp repeats (plus 13 bp of a fifth repeat) are bracketed. The underlined regions indicate the five restriction sites used in the RFLP analyses (restriction sites in bold). Note that a T→A polymorphism at nt 167, not present in the 7 samples shown here, created an additional *Dpn*II site in 3 nuclear-transfer sheep samples.

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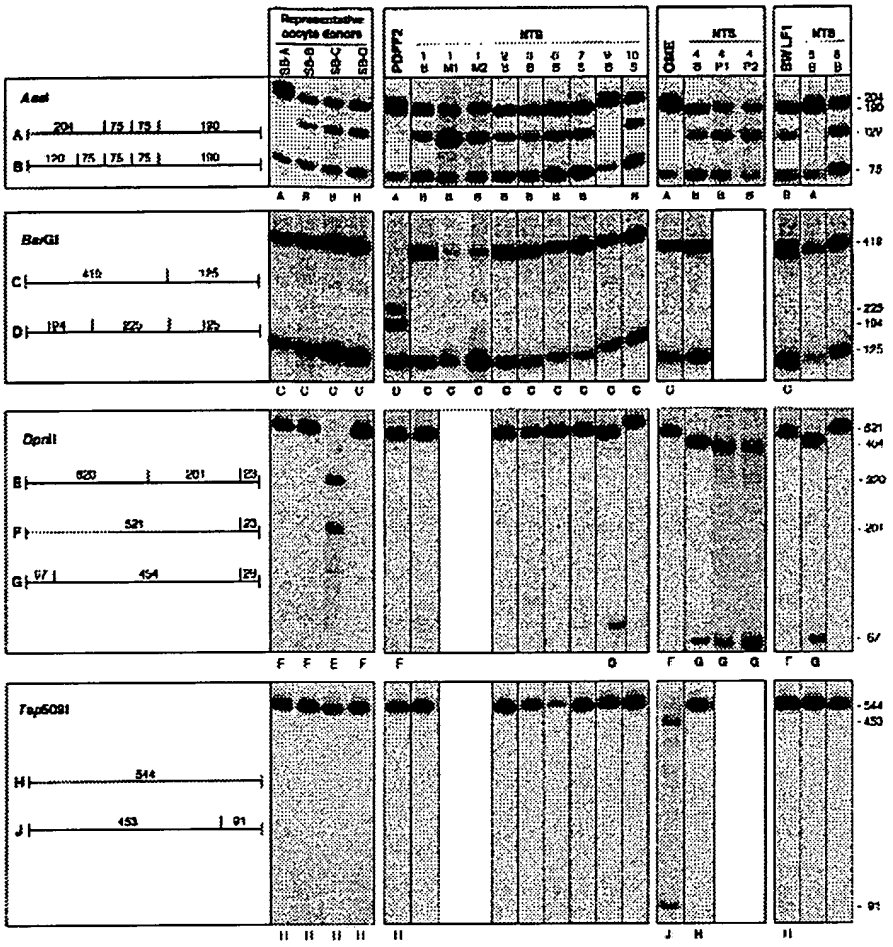

Fig. 2 PCR/RFLP analyses of the 544-bp PCR-amplified D-loop fragments, showing the maps with the predicted digestion fragment sizes, in bp, for each of the 4 indicated restriction endonucleases. Autoradiograms of the respective gels are to the right of each set of maps. The first group of autoradiograms (nearest the maps) shows the RFLP patterns in the four representative oocyte donor samples. In each of the three other groups of autoradiograms, the first lane shows the RFLP pattern in the nuclear donor (bold), followed by the pattern in the nuclear-transfer sheep (NTS) derived from that donor; the tissue analysed (B, blood; S, skeletal muscle; M1, M2, milk (replicates); P1, P2, placenta (replicates)) for each sample (numbered) is indicated above each NTS lane. The mtDNA genotypes (A–J) are indicated at left. Genotypes are shown below the oocyte and donor lanes; only the genotypes below the NTS lanes that were informative for the origin of the indicated mtDNA genotype are shown (bold). Gel fragment sizes in bp are indicated (right).

into the fetus contains approximately 1,000 copies of mtDNA. Third, during normal sexual reproduction, mitochondria (and mtDNAs) are maternally transmitted[12]; paternal mitochondria enter the oocyte, but are eliminated rapidly by an unknown mechanism during the first few zygotic cell divisions[13,14]. Fourth, although an individual member of a species normally harbours a single mitochondrial genotype (homoplasmy), an animal can harbour two mtDNA genotypes (heteroplasmy) if a mutation in the germ line of the mother passes into the progeny. Finally, any two genetically unrelated homoplasmic individuals differ at approximately 0.3% of the nucleotides in their mtDNAs, with most mutations located in the highly polymorphic D-loop region (which is the control region in mtDNA that is devoid of structural genes).

There are three possible outcomes in animals cloned by nuclear transfer via whole-cell electrofusion: homoplasmy of donor somatic cell mtDNA; homoplasmy of recipient oocyte mtDNA; or heteroplasmy due to mixing of donor and recipient mtDNAs. The determination of the mitochondrial character of cloned sheep required the analysis of mtDNA from three sources: (i) the nuclear donor somatic cells; (ii) the recipient oocyte; and (iii) the nuclear transfer-derived cloned sheep. We used DNA sequencing of the D-loop region and PCR/RFLP analysis based on polymorphisms found between donor and recipient samples to differentiate the mtDNA genotypes (both qualitatively and quantitatively) among the three relevant sources.

We analysed DNA from three donor cell types: OME, an adult mammary gland primary epithelial cell culture[5]; and PDFF2 and BLWF1 (ref. 6), fetal fibroblast primary cell cultures derived from day-35 and day-25 fetuses, respectively. We also analysed DNA from tissues (blood, skeletal muscle, placenta and milk) obtained from ten nuclear transfer-derived sheep, including Dolly (we confirmed that the nuclear DNA in all the samples matched the nuclear DNA isolated from the appropriate somatic donor cells; data not shown). Unfortunately, the animal husbandry regime, the practicalities of the oocyte collection regime and the use of intermediate recipients precluded identification of the Scottish Blackface (SB) oocyte donor for any individual nuclear-transfer lamb. (During each nuclear-transfer session, oocytes derived from eight individual ewes were pooled. Following recovery, the donor ewes were sold to a slaughterhouse. No tissue samples were retained for specific comparison.) To overcome this problem, we analysed the D-loop region (Fig. 1*a*) of tissues from four randomly selected SB sheep as a representative sample. A 544-bp segment of the 1.2-kb sheep D-loop[15] from OME, PDFF2 and BLWF1 donor cells and from the SB samples was amplified by PCR from total genomic DNA and sequenced (Fig. 1*b*). Relative to a reference D-loop sequence[15], we found 40 polymorphisms, 4 of which generated useful restriction endonuclease recognition sites (Fig. 1*b*).

On RFLP analysis, all somatic donor cell and representative oocyte-recipient SB samples displayed the predicted patterns (Fig. 2). The same region of the D-loop was amplified from the DNA of the ten nuclear transfer-derived sheep. RFLP analysis of these amplified products showed that all ten were homoplasmic for the mitochondrial genotype of the SB recipient oocyte in all

tissues examined, with no evidence for the presence of donor mitochondria-derived mtDNAs (Fig. 2).

An A→G polymorphism at nt 223 (Fig. 1*b*) destroys an *Ase*I site in OME (genotype A; Fig. 2). This *Ase*I site is present in OME-derived sample 4 (Dolly; genotype B). Conversely, nuclear transfer-derived sheep 5 is genotype A, whereas its nuclear donor BLWF1 is genotype B. Similarly, samples 1, 2, 3, 6, 7 and 10 are genotype B, whereas their nuclear donor PDFF2 is genotype A. The *Ase*I genotypes of nuclear transfer-derived sheep 8 and 9 were uninformative, as the same genotypes were present in both their respective nuclear donors and the SB samples.

A C→T polymorphism at nt 288 (Fig. 1*b*) creates an additional *Bsr*GI site in the amplified D-loop region of PDFF2 (genotype D; Fig. 2). PCR/RFLP analysis of PDFF2 gave the expected restriction pattern with this enzyme (Fig. 2). All seven nuclear transfer-derived sheep derived from PDFF2 (1, 2, 3, 6, 7, 9 and 10; Fig. 2) showed no evidence of this polymorphism (all had genotype C).

A T→C polymorphism at nt 418 (Fig. 1*b*) creates a new *Dpn*II site (genotype E; Fig. 2) in one of four representative oocyte recipients (SB-C), but this specific site is not present in the other three oocyte recipients or the three nuclear donors (that is, they are genotype F). Of the ten nuclear transfer-derived sheep, seven were also genotype F, whereas three (sheep 4, 5 and 9) contained a different informative polymorphism (T→A at nt 167, creating a *Dpn*II site; genotype G) not present in donor or oocyte samples (Fig. 1*b*). Each of these three sheep was derived from a different nuclear donor (OME, BLWF1 and PDFF2, respectively), but none of the donors had genotype G. This indicates that the mtDNA genotypes of the nuclear transfer-derived sheep do not match those of their nuclear donors (presumably, genotype G is present in the SB population, although it was absent in the four SB samples analysed here).

Finally, sequencing of the OME-derived PCR fragment revealed a C→T polymorphism at nt 550 (Fig. 1*b*) that creates a new *Tsp*509I site (genotype J; Fig. 2). Dolly (Fig. 2, sheep 4) is derived from the OME cell line, yet showed no evidence of this mtDNA polymorphism (Fig. 2, genotype H).

We performed 48 RFLP analyses, of which 26 yielded informative results (that is, we were able to distinguish the mtDNA genotype of the somatic donor cells from that of the representative oocyte-recipient sheep breed). All 26 indicated that the nuclear transfer-derived sheep were homoplasmic for the oocyte recipient mtDNA, and none of the 22 non-informative RFLPs contradicted this conclusion. We estimate that the limit of detection in our methodology was at least 99.5–99.9%. We based this range on quantitations performed using three different methods.

The finding of mtDNA homoplasmy in all ten nuclear transfer-derived sheep was unexpected. Because the nuclear transfer method via electrofusion almost certainly introduces donor cytoplasm, including mitochondria, into the cytoplasm of the recipient SB oocyte[7,8], one would expect to observe at least some contribution of donor-derived mtDNA in the clones (heteroplasmy). To estimate the lower limit of the potential contribution of mtDNA from donor cytoplasm, we quantitated the number of mtDNAs in OME cells by dot blot analysis (data not shown). We estimated that OME cells contain 2,000–5,000 mtDNAs per cell, values only slightly lower than those in transformed human somatic cells[11]. Although we did not analyse the BLWF1 and PDFF2 donor cells, we assume they had similar amounts of mtDNA. The mtDNA composition of each fetal sheep derived by nuclear transfer should reflect that of the original electrofused donor/recipient cell—assuming that the inheritance of somatic donor mtDNAs is similar to that of maternal germline mtDNAs, and that there was homogeneity of cytoplasmic mixing—in which case we should have observed 2–5% of donor-derived heteroplasmic mtDNA in the samples.

Our failure to detect donor mtDNAs in any of the examined tissues from the nuclear transfer-derived sheep implies that random partitioning of mtDNAs (ref. 16) did not occur. This may be due to the failure of the donor mitochondria to enter the ooplasm following electrofusion, to skewed segregation of donor mtDNAs during the cloning process or to some unknown effect of maintaining donor cells in G_0 for five days before nuclear transfer[5]. We favour a scenario in which an active mechanism operates to destroy the donor mitochondria in the recipient ooplasm, similar to what is thought to happen to sperm-derived mitochondria in fertilized ova in both normal human reproduction[12,14] and in intraspecific (but not in interspecific[13,17,18]) mouse crosses[13,19].

Our conclusion that the nuclear transfer-derived sheep were homoplasmic is based on the analysis of specific tissues in each animal. We analysed one tissue (either blood or skeletal muscle) from each animal, and in two animals we also analysed a second tissue type (milk from sample 1 and placenta from sample 4, Dolly). One or two tissues, however, may not accurately reflect the mtDNA composition of the entire animal. For example, wide variations in heteroplasmy were detected in different progeny and in different tissues of mice produced from fertilized eggs containing exogenous oocyte-derived mitochondria generated by pronuclear karyoplast fusions with enucleated zygotes[20,21] or injections of heterologous oocyte cytoplasm into recipient oocytes[21–23]. In addition, heteroplasmic patients harbouring pathogenic mutations in human mtDNA (ref. 24) sometimes contain significantly different proportions of the mutation in different tissues, as in mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), where the proportion of mutated mtDNA in blood is usually lower than that in other tissues[25]. In other mitochondrial disorders, however, such as myoclonus epilepsy with ragged-red fibers[26] (MERRF) and maternally inherited Leigh syndrome[27] (MILS), the mtDNA mutation is distributed relatively homogeneously in all tissues. Of the sheep samples examined, we obtained three (2, 7 and 10) from skeletal muscle, a long-lived post-mitotic tissue which might be more likely to exhibit heteroplasmy, if present in the animal[24]. Our conclusions, however, are tempered by the fact that we were unable to obtain samples of other sheep tissues, such as brain and heart, that are frequently affected in human mitochondrial diseases[24]. Although we cannot eliminate the possibility that there is heteroplasmy in other unexamined tissues of the cloned animals, the uniformity of the results from multiple sample sources supports the conclusion that the clones were homoplasmic for oocyte-derived mtDNA.

These results have implications for future attempts to correct maternally inherited mitochondrial genetic disorders by, for example, nuclear transfer involving a somatic or germline cell from a woman harbouring a pathogenic mtDNA mutation (but normal nuclear DNA) and a recipient enucleated oocyte[28,29] (containing normal cytoplasm). If the experience with cloned sheep is any guide, we would predict that the human mitochondrial genotype will be determined by the recipient ooplasm.

Methods

Sheep samples. We isolated total DNA from three somatic nuclear donors (OME cultured mammary gland cells derived from a Finn Dorset sheep[5]; BLWF1 fibroblast cells derived from a day-25 Black Welsh Mountain fetus[6]; and PDFF2 fibroblast cells derived from a day-35 Poll Dorset fetus[6]); four representative Scottish Blackface sheep (SB-A, -B, -C and -D); and ten nuclear transfer-derived sheep[5,6]. Nuclear transfer sheep 1, 2, 3, 6, 7, 9 and 10 were from PDFF2; 5 and 8 were from BLWF1; and 4 (Dolly) was from OME. We isolated DNA samples from blood (sheep 1, 3, 4, 5, 6, 8 and 9), tongue muscle (2, 7 and 10), milk (1) and placenta (4).

D-loop analyses. We amplified a 544-bp region of the sheep D-loop[15] with forward primer 96F (nt 96–113; ref. 15) and reverse primer 639B (nt 639–621) with *Taq* DNA polymerase (Perkin Elmer). Conditions were 94 °C for 30 s, 66 °C for 30 s and 72 °C for 30 s, for 30 cycles, followed by 7 min at 72 °C, performed on a model 9700 thermocycler (Perkin Elmer). We sequenced the PCR products using the fmol system (Promega). We performed multiple amplifications from the original samples to minimize the possibility of identifying PCR-induced mutations; none were detected.

RFLP analyses. We amplified samples by PCR in the presence of [α-^{32}P]dCTP, digested them with appropriate enzymes and electrophoresed the digested products through 8% non-denaturing polyacrylamide gels. After drying the gels, we visualized and quantitated the labelled fragments in a phosphorimager (BioRad Model GS363) or on X-ray film. We estimated the limit of detection for potential heteroplasmy using three different methods: (i) RFLP analysis of PCR-amplified mixtures of two known genotypes was able to distinguish heteroplasmy to a dilution of 1:200 (that is, detection limit of at least 99.5%); (ii) RFLP analysis of PCR fragments (amplified from skeletal muscle DNA isolated from clone sample 2 (PDFF2)) subcloned into pCR2.1 (Invitrogen) showed only a single genotype in 190 randomly picked clones (that is, detection limit of at least 99.5%); and (iii) phosphorimager quantitation of the ratio of the signal derived from an authentic RFLP fragment to that of the signal located in the region of the gel in which a predicted heteroplasmic fragment should appear was ~1,000:1 (that is, detection limit of 99.9%), and was confirmed by measuring the signals from known serial dilutions of two labelled DNA samples.

Quantitation of mtDNA in somatic cells. We used serial dilutions of the PCR-amplified sheep D-loop region as standards in dot blot analyses of serial dilutions of OME-derived total DNA isolated from a known number of OME cells. We probed the blots with the sheep D-loop region, labelled by random priming and quantitated the dot intensities as above. The D-loop probe detected only authentic mtDNA, as confirmed by the detection of a single 16.5-kb hybridizing band in Southern-blot analysis of *Pst*I-digested sheep total DNA (data not shown).

Microsatellite analysis. We used ovine nuclear microsatellite marker pairs (5′ and 3′) MAF33, FCB304, FCB11 and MAF209 to establish the nuclear genotype of the various samples, as described[30].

Acknowledgements

We thank E.A. Shoubridge, M. Hirano and S.C. Silverstein for helpful discussions. This work was supported by grants from the National Institutes of Health (NS28828; NS11766; HD32062; HL52145) and the Muscular Dystrophy Association.

Received 24 February; accepted 7 July 1999.

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